Protease inhibitor from *Moringa oleifera* leaves: Isolation, purification, and characterization

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ABSTRACT

Protease inhibitors have great demand in medicine and biotechnology. We report here the purification and characterization of a protease inhibitor isolated from mature leaf extract of *Moringa oleifera* that showed maximum inhibitor activity. The protease inhibitor was purified to 41.4-fold by Sephadex G75 and its molecular mass was calculated as 23,600 Da. Inhibitory activity was confirmed by dot-blot and reverse zymogram analyses. Glycine, glutamic acid, alanine, proline and aspartic acid were found as the major amino acids of the inhibitor protein. Maximal activity was recorded at pH 7 and at 40 °C. The inhibitor was stable over pH 5–10; and at 50 °C for 2 h. Thermostability was promoted by $CaCl_2$, BSA and sucrose. Addition of Zn^{2+} and Mg^{2+}, SDS, dithiothreitol and β-mercaptoethanol enhanced inhibitory activity, while DMSO and H_2O_2 affected inhibitory activity. Modification of amino acids at the catalytic site by PMSF and DEPC led to an enhancement in the inhibitory activity. Stoichiometry of trypsin–protease inhibitor interaction was 1:1.5 and 0.6 nM of inhibitor effected 50% inhibition. The low K_i value (1.5 nM) obtained indicated scope for utilization of *M. oliefera* protease inhibitor against serine proteases.

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1. Introduction

Recently enzyme inhibitors have received greater significance owing to their potential as useful tools for the study of enzyme structures and reaction mechanisms, and their utilization in pharmacology and agriculture [1–3]. Specific and selective protease inhibitors are powerful tools for inactivating target proteases in the pathogenic processes of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer, and AIDS. Protease inhibitors which specifically inhibit the proteases that are essential in the life cycle of organisms that cause mortal diseases such as malaria, AIDS, and cancer can be used in drug design towards prevention of propagation of these causative agents [4]. Protease inhibitors can be employed effectively as defense tools by virtue of their antinutritional interaction against insects that possess alkaline guts and depend predominantly on serine proteases for digestion of plant material [5]. Microbial food spoilage is yet another area of global concern and it has been estimated that as much as 25% of all food produced is lost post-harvest owing to microbial activity [6].

Application of natural protease inhibitors could be an effective way to prevent proteolysis and extend the shelf life of seafood, since the inhibitors can retard proteolysis caused by the action of endogenous and exogenous proteases during food processing and preservation [7].

Plants were recognized as a potential source for most of the naturally occurring protease inhibitors which have been isolated and well characterized. Most of them were found to belong to the group of serine protease inhibitors which could inhibit trypsin and chymotrypsin [8,9]. Although a large number of protease inhibitors have been isolated and identified from several plants [9], *Moringa oleifera* as source of protease inhibitor is not yet reported. *M. oleifera* is a panotropical multipurpose tree with a high biomass yield and capable of tolerating unfavorable environmental conditions [10]. It grows throughout the tropics and almost every part of the plant is of value as food. The flowers, leaves, and roots are used in folk remedies for tumors and the seeds for abdominal tumors. Bark regarded as antiscorbic exudes a reddish gum with properties of tragacanth which is used for the treatment of diarrhea. Roots are bitter and act as a tonic to the body and lungs. Further roots are also used as expectorant, mild diuretic, and stimulant in paralytic afflictions such as epilepsy and hysteria [11].

The leaves of *M. oleifera* are enriched with vitamin A and C. Pods and young leaves of the plants are primarily used for vegetative purpose. In addition, isolation of several low molecular weight bioactive compounds from the seeds with bactericidal, fungicidal,

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and anti inflammatory activities [12,13], bioactive nitrile glycosides niaziridin and niazirin from the leaves, pods and bark [14,15], bioinsecticidal coagulant lectin [16], and immunosuppressive compounds in seeds [17] were reported. In this context the objective of our investigation was to screen various parts of *M. oleifera* for protease inhibitors towards their probable utilization in development of thereapeutic drugs, biocontrol agents and as food preservative against proteolysis. Herein we report the isolation, purification, and characterization of protease inhibitors from *M. oleifera* leaves.

2. Materials and methods

2.1. Plant materials

M. oleifera leaves, flowers, seeds, and barks were collected from nearby areas around Cochin University of Science and Technology campus, India. The plant materials were washed thoroughly in distilled water, air dried and ground to powder using an electrical blender.

2.2. Extraction and recovery of protease inhibitor

Extractant that supported maximal extraction of the protein protease inhibitor from the plant materials was selected after standardization of the extraction protocol with different solutions: viz NaCl 15% (w/v), NaOH 0.2% (w/v), HCl 0.05 M [18], phosphate buffer 0.1 M (pH 7.0) [19] and deionized water. Extraction was carried out by homogenizing 25 g of sample in 100 mL of the extracting solution in an electrical blender. The prepared homogenate was incubated at room temperature (RT, $28 \pm 2\,^\circ$C) on a rotary shaker for 30 min at 150 rpm. Later the slurry was filtered through cheesecloth and the filtrate was centrifuged at 10,000 rpm for 15 min at 4 °C for the removal of any cell debris that remained in the preparation [20]. The clear supernatant obtained was used as the crude extract for the assay of protease inhibitor activity, protein content, and specific activity as described below.

2.3. Protease inhibitor assay

Activity of protease inhibitor was assayed according to the method of Kunitz with slight modifications against trypsin [21]. One millilitre aliquot of trypsin (EC 3.4.21.4, SRL, India) (0.5 mg/mL equal to 500 units/mg) prepared in phosphate buffer (0.1 M, pH 7) was pre incubated with 1 mL of the protease inhibitor at 37 °C for 15 min. To the above mixture 2 mL of 1% Hammerstein casein (SRL, India), prepared in 0.1 M phosphate buffer, was added and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 2.5 mL of 0.44 M trichloroacetic acid (TCA) solution. The reaction mixture was centrifuged at 10,000 rpm for 15 min (Sigma, Germany) and the absorbance of the clear supernatant was measured at 280 nm in a UV–visible spectrophotometer (Shimadzu, Japan) against appropriate blanks. The TCA soluble peptide fractions of casein formed by the action of trypsin in the presence and absence of the inhibitor were quantified by comparing with tyrosine as standard. One unit of trypsin activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per millilitre of the reaction mixture per minute under the assay conditions. One unit of protease inhibitor activity (PIU) was defined as the decrease by one unit of absorbance of TCA soluble casein hydrolysis product liberated by trypsin action at 280 nm per minute under the assay conditions. For easy computation and understanding, the protease inhibitor activity was expressed in terms of percent inhibition of trypsin activity throughout the course of study. Appropriate blanks for the enzyme, inhibitor, and the substrate were also included in the assay along with the test.

2.4. Protein estimation

Protein content was determined according to the method of Lowry et al. [22] using bovine serum albumin (BSA) as the standard and the concentration was expressed in milligram per millilitre (mg/mL).

2.5. Purification of protease inhibitor

The clear supernatant obtained after extraction, homogenization, and centrifugation was fractionated by 30–90% ammonium sulphate [(NH$_4$)$_2$SO$_4$] saturation [23] and the resultant precipitates were dialysed against phosphate buffer (0.01 M, pH 7). The dialysate was dissolved in a small amount of 0.05 M Tris–HCl buffer (pH 7.6) and 5 mL of this crude inhibitor preparation was applied onto a DEAE cellulose [24] column (XK16/26 column, Amersham Pharmacia). The proteins were eluted using citrate buffer (pH 3) selected based on preliminary standardization experiments (data not shown) and fractions (5 mL/fraction) were collected at a flow rate of 0.5 mL/min. Fractions corresponding to protease (trypsin) inhibitory activity were pooled and applied onto a Sephadex G75 column (Amersham Pharmacia XK 26/70 column) and proteins were eluted using citrate buffer (pH 3), and fractions (5 mL/fraction) were collected at a flow rate of 0.4 mL/min.

Active fractions obtained after gel filtration were lyophilized as 1 mL aliquots and resuspended in 0.1 mL of the sample buffer (0.0625 M Tris–HCl, 10% sucrose, 0.01% bromophenol blue, pH 6.8). Samples were loaded on to a preparative polyacrylamide gel and subjected to electrophoresis [25] in a vertical slab electrophoresis apparatus (dual mini vertical electophoretic unit-Tarsons, India) using low molecular weight markers (Amersham Pharmacia) as standard. After electrophoresis, a portion of the gel with marker was stained and compared with the original gel. The protein bands were eluted using an Electro elutor (FINE PCR Electro elutor) (25 V, 10 min, 4 °C) into 200 μL of Tris buffer (pH 8.3) and dialyzed against phosphate buffer (0.01 M, pH 7). The purified protein samples were then lyophilized.

2.6. Characterization of protease inhibitor

2.6.1. Analysis of protease inhibitor by polyacrylamide gel electrophoresis, reverse zymography, and dot-blot method

The purified protease inhibitor was subjected to electrophoretic analysis by Native-PAGE and SDS-PAGE in a vertical slab electrophoresis apparatus (dual mini vertical electophoretic unit-Tarsons, India) using 10% polyacrylamide gel [25]. Protease inhibitor activity of the purified protein was confirmed by reverse zymogram on gelatin-PAGE performed by adding gelatin (0.1% final concentration) to the polyacrylamide gel [26]. The purified fractions were further analyzed for their protease inhibitor activity by dot-blot method [27].

2.6.2. Amino acid analysis

Amino acid analysis of the purified inhibitor protein sample was done [28] using Shimadzu High-Performance liquid chromatography (LC-4A) "Amino Acid Analysis System".

2.6.3. Effect of pH on protease inhibitor activity and stability

Optimum pH for the maximal activity of the protease inhibitor was determined by performing protease inhibitor assay at different pH ranging from 2 to 12. The substrate casein (1%) was prepared in the respective pH buffers which included glycine–HCl (pH 2–3.5), citrate (pH 4–6), phosphate (pH 6–8), Tris–HCl (pH 8–9), carbonate–bicarbonate (pH 9–10.5), boric acid/KCl/NaOH (pH 11), and Na$_2$HPO$_4$/NaOH (pH 12).

The stability of protease inhibitor over a range of pH was determined by evaluating the inhibitor activity at pH 7 after incubating the purified protease inhibitor in buffers of different pH (2–12) for 24 h, at 4 °C. Purified inhibitor (1.2 mL) was incubated with 10.8 mL of different buffer systems mentioned above. After incubation, 1 mL of the sample was assayed for protease inhibitor.

2.6.4. Effect of temperature on protease inhibitor activity and stability

Optimum temperature for the maximal activity of protease inhibitor was determined by assaying the inhibitor activity at different incubation temperatures (10–80 °C). Further, stability of the purified inhibitor at different temperatures was also evaluated by incubating 1.2 mL of the purified protease inhibitor at different temperatures ranging from 30 to 70 °C. The samples were drawn at different time intervals: 30 min, 1 h, 2 h, 4 h, 8 h and 12 h, further incubated at 4 °C for 30 min, and assayed for protease inhibitor activity.

2.6.5. Effect of stabilizers on thermal stability of protease inhibitor

The enhancement of thermal stability of protease inhibitor at 50 °C and 60 °C was evaluated by the addition of thermal stabilizers like glycine (1 M), cysteine hydrochloride (10 mM), PEG 8000 (10 mM), glycerol (10%), sorbitol (10%), casein (1%), CaCl$_2$ (10 mM), urea (10 mM), sucrose, BSA, and starch (at 1% level) (arbitrarily selected based on available literature on protease). Samples were drawn at regular intervals of 1 h, 2 h, 4 h and 8 h, incubated at 4 °C for 30 min, and assayed for protease inhibitor activity.

2.6.6. Effect of various metal ions on protease inhibitor activity

Effect of various metal ions on activity of protease inhibitor was evaluated using sodium chloride, calcium chloride, magnesium sulphate, zinc sulphate, cupric sulphate, ferric chloride, manganese chloride, nickel chloride, mercury chloride, barium chloride, cadmium sulphate, sodium molybdate, and aluminum chloride that contribute the metal ions respectively Na$^+$, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Ba^{2+}, Cd^{2+}, Mo^{6+} and Al^{3+} each at 1 and 10 mM final concentrations in the reaction mixture. The protease inhibitor was added to different concentrations of metal ions, incubated for 30 min at 37 °C, and assayed for protease inhibitory activity.

2.6.7. Effect of surfactants, oxidizing agents, and reducing agents on protease inhibitor activity

Effect of various non-ionic and ionic surfactants such as Triton X-100, SDS, Tween-80, Tween-20, and Brij-35 on protease inhibitor activity was determined by incubating the protease inhibitor with each surfactant for 30 min followed by dialysis of the mixture against phosphate buffer (0.01 M, pH 7) and estimation of protease inhibitor activity. Impact of oxidizing agents [H$_2$O$_2$ and dimethyl sulfoxide (DMSO)] at concentrations of 1, 2, 3, 4 and 5% (v/v) and reducing agents [dithiothreitol and β-mercaptoethanol at concentrations of 0.2, 0.4, 0.6, 0.8 and 1% (v/v)] on the activity of protease inhibitor was studied by incubating the protease inhibitor in the presence of them for 30 min and then measuring the protease inhibitor activity.
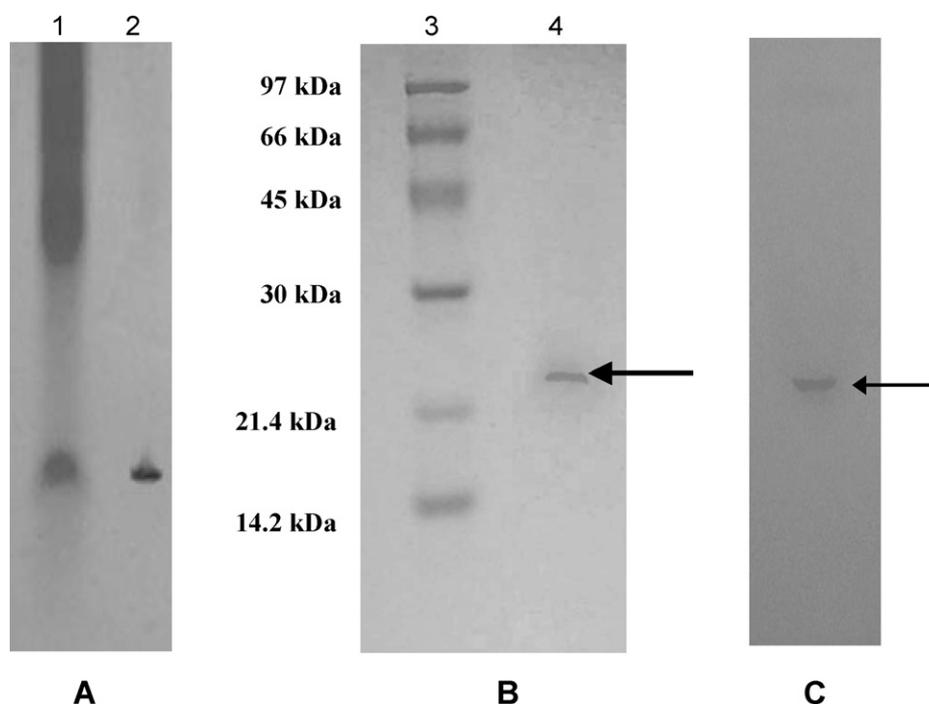
Fig. 1. Native-PAGE (A) and SDS-PAGE (B) analysis of *Moringa oleifera* protease inhibitor: samples were run on a 10% polyacrylamide gel and stained with commassie brilliant blue. Lane 1, crude extract; lane 2, purified protease inhibitor; lane 3, low molecular weight markers; and lane 4, purified protease inhibitor; activity staining of purified protease inhibitor (C): reverse zymography – purified sample was run on gelatine PAGE.

2.6.8. Effect of chemical modifications of amino acids at the reactive sites on protease inhibitor activity

Chemical modifications of the amino acids (lysine, histidine and serine) of the purified inhibitor were carried out using different chemical modifiers succinic anhydride [29], diethyl pyrocarbonate (DEPC) [30], and phenylmethylsulfonyl fluoride (PMSF) [31] which modifies the amino acids lysine, histidine and serine, respectively, under their respective reaction conditions. An aliquot of 2 mL of the purified inhibitor (2.7 mg/mL) was incubated with different concentrations (5, 10, 15, 20 and 25 mM) of each modifier, dialyzed against phosphate buffer and estimated the protease inhibitor activity.

2.6.9. Stoichiometry of protease–protease inhibitor interaction, IC_{50} value of protease inhibition and kinetic studies

The molar concentration of the purified protein protease inhibitor for complete inactivation of trypsin was determined by preincubating 1 nM trypsin (based on M_r 23,800) in 100 μL of phosphate buffer (0.1 M, pH 7) with different amounts of chromatographically purified protease inhibitor (0.25–2.0 nM, based on M_r 23,600) at 37 °C for 60 min. The remaining activity of trypsin was determined by the addition of 1% casein, followed by incubation and spectroscopic examination according to Kunitz method [21]. The amount of protein protease inhibitor needed for 50% inhibition of protease activity was determined by conducting the protease inhibitor assay as described above.

Kinetic studies on protease inhibitor were conducted using trypsin. Using the enzyme rate data a double reciprocal-plot was prepared and analyzed to determine whether the nature of protease inhibition is competitive, uncompetitive or non-competitive. Protease inhibition kinetics was studied using various concentrations of substrate ranging from 0.001 to 2.0 mM [32]. An aliquot of 500 μL of 1 nM trypsin alone was preincubated in 0.1 M phosphate buffer for 20 min at 37 °C. Fractions of 500 μL of different concentrations (4, 6, and 8 nM) of purified protease inhibitor were preincubated with aliquots of 100 μL of 1 nM trypsin for 20 min at 37 °C. Later the pre-incubated mixtures were added separately to 0.001–2.0 mM Nα-benzoyal-arginine-4-nitroanilide (BAPNA) (Sigma–Aldrich) solution and incubated at 37 °C for 10 min. After incubation the reaction was arrested by adding 200 μL of 30% (v/v) acetic acid. The liberated p-nitro aniline was measured at 410 nm in a UV–visible spectrophotometer (Shimadzu, Japan). One protease unit was defined as the amount of enzyme that increased absorbance by 1 OD/min and one protease inhibitor unit is defined as the amount of protease inhibitor that inhibited one unit of protease activity. Protein inhibitor activities were finally expressed as percent inhibition.

The velocity of the enzymatic reaction (v) based on the rate of change in absorbance (A_{410}) of the reaction mixture was determined for each concentrations of BAPNA used. The initial velocity data was plotted as a function of the concentration of substrate by the linear transformation of the Michaelis–Menten equation and usual non-linear curve fitting of the Michaelis–Menten equation for the calculation of K_m and V_{max} of the reaction. A Lineweaver–Burk curve, $1/v$ versus $1/[s]$, was plotted to study the pattern of inhibition (competitive, uncompetitive or non-competitive). A secondary plot was drawn by $1/K'_m$ versus concentrations of inhibitor studied. The X-intercept gives the $-K_i$ value and from that the dissociation constant (K_i) was calculated [33].

3. Results

3.1. Isolation of protease inhibitor from different parts of M. oleifera

Among the various extraction media evaluated for recovering protease inhibitor molecules from *M. oleifera*, the crude extract prepared in phosphate buffer showed maximum protease inhibitor activity (79% inhibition) followed by distilled water (68% inhibition). Among the different parts of the *M. oleifera* screened, mature leaves (77%) and seeds (63%) recorded high levels of inhibition against trypsin activity. Barks, flowers, and roots however recorded very less amount of trypsin inhibitor activity (data not shown). Hence, leaves of mature *M. oleifera* plant were used for further studies.

3.2. Purification of the protease inhibitor

The yield and fold of purification of protease inhibitor from the leaves of mature *M. oleifera* is summarized in Table 1. It was found that 60–90% $(NH_4)_2SO_4$ (w/v) saturation was efficient for precipitating the protease inhibitor compared to other fractions. The fold of purification of protease inhibitor obtained for $(NH_4)_2SO_4$ precipitation, ion exchange chromatography, and gel filtration chromatography were 1.5, 2.5, and 41.4, respectively. The binding pH of the protein to the DEAE cellulose was standardized to acidic pH 3. A single protein peak demonstrating maximum inhibitory activity towards trypsin was eluted at 0.2 M NaCl in citrate buffer (pH 3) which resulted in an increase in purification fold up to 2.5 with a specific inhibitory activity of 32.8 units/mg protein.

The elution profile of protease inhibitor obtained from gel filtration chromatography using Sephadex G75 column (data not shown)

Table 1
Yield and fold of purification of protease inhibitor isolated from *M. oleifera* leaves.

Sample	Volume (mL)	Total protein (mg)	Protease inhibitor activity (PIU)	Specific inhibitor activity (PIU/mg)	Yield of activity (%)	Fold of purification
Crude extract	100	710	9400	13.2	100	1
(NH$_4$)$_2$SO$_4$ fraction (30–90%)	36	126	2520	20	26.8	1.5
Ion exchange fraction (DEAE)	56	116	3808	32.8	40.5	2.5
Gel filtration by Sephadex G75	0.5	0.095	52	547.4	0.6	41.4

resulted in 41.4-fold of purification and showed a single major peak indicating the purity of the protease inhibitor protein. When analyzed subsequently through Native-PAGE, a single protein band with 52% protease inhibitor activity confirming their purity and homogeneity (Fig. 1A) was obtained.

3.3. Characterization of protease inhibitor

3.3.1. Molecular mass determination, reverse zymography, and dot-blot analysis

The molecular mass of the protein protease inhibitor was estimated based on SDS-PAGE analysis. Single polypeptide bands with a molecular mass of 23,600 Da in non-reductive SDS-PAGE (Fig. 1B) testified the purity of the fraction. Results observed for the reverse zymogram analysis on gelatin-PAGE testified the protease inhibitor activity of the molecule (Fig. 1C). Dot-blot analysis of protease inhibitor activity, performed on X-ray film, indicated that the protease inhibitor blocked the gelatin hydrolysis by trypsin. The presence of inhibitor was confirmed by comparing the clearing zone formed due to gelatin hydrolysis by trypsin and a reduction in clearing zone by trypsin incubated with protease inhibitor (Fig. 2).
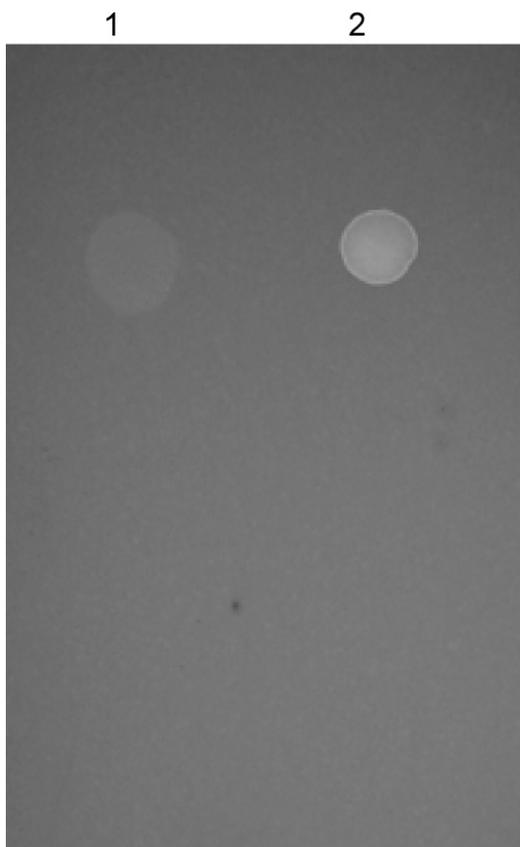

Fig. 2. Dot-blot analysis of purified protease inhibitor spotted on X-ray film. (1) *M. oleifera* inhibitor with trypsin and (2) trypsin alone.

Table 2
Amino acid composition of *Moringa oleifera* protease inhibitor (expressed as g amino acid/100 g of protein).

Amino acid	%
Glycine	27.29
Glutamic acid	12.53
Alanine	11.19
Proline	10.74
Aspartic acid	8.95
Valine	5.15
Serine	4.25
Leucine	4.25
Arginine	4.02
Threonine	3.36
Isoleucine	3.13
Phenyl alanine	2.23
Histidine	1.79
Methionine	0.89
Lysine	0.22

3.3.2. Amino acid analysis

From the results presented in Table 2 it was noted that glycine (27.29%) was the major constituent followed by glutamic acid (12.53%), alanine (11.19%), proline (10.74%) and aspartic acid (8.95%). Compared to these, other amino acids were present in lesser amounts. Valine, serine, leucine, arginine, threonine, isoleucine, phenylalanine, histidine, methionine, and lysine were recorded in the decreasing order of concentration in the protease inhibitor protein in the range of 5.15–0.22%.

3.3.3. Effect of pH on the activity and stability of protease inhibitor

Data presented in Fig. 3 indicated that the protease inhibitor was active over a wide range of pH 6–10 recording maximal inhibitor activity at pH 7 (68% inhibition) compared to that noted at other pH levels. Further, it was observed that the inhibitor lost its activity at highly acidic and highly alkaline pH conditions. Stability studies
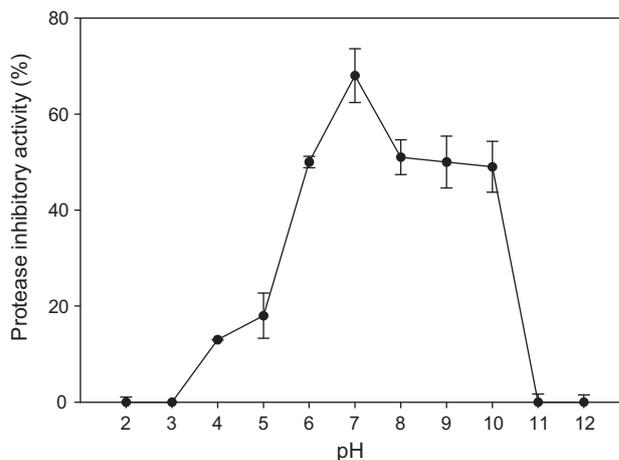

Fig. 3. Activity profile of the protease inhibitor at different pH: protease inhibitor assay was conducted with casein prepared in different buffer systems of pH 2–12 at 37 °C.
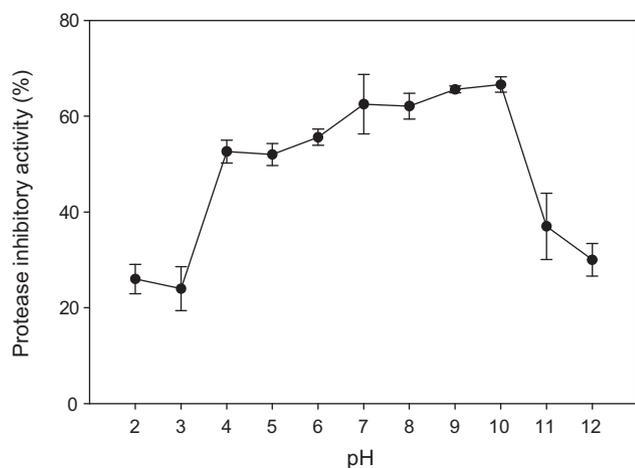
Fig. 4. Stability of protease inhibitor at different pH: activities of protease inhibitor incubated at pH 2–12 for 24 h were measured at pH 7 and 37 °C.

Fig. 6. Stability studies of protease inhibitor at different temperatures: *M. oleifera* protease inhibitor incubated at different temperatures were drawn at different time intervals and assayed for inhibitor activity at pH 7 and 37 °C.

conducted for a period of 24 h in different buffer systems showed that the protease inhibitor was stable over a wide range of pH (5–10) with maximal inhibitory activity retained at pH 10 (67% inhibition) (Fig. 4). However, at highly acidic (pH 3) and at alkaline (pH 11) conditions the inhibitory activity sharply declined recording 16% and 37% inhibitions, respectively, compared to the inhibitory activity recorded at pH 10.

3.3.4. Effect of temperature on the activity and stability of protease inhibitor

The protease inhibitor was observed to be most active only up to 50 °C recording a maximal of 83% protease inhibition in the range of 30–40 °C (Fig. 5). It was noted that the protease inhibitor activity declined at temperatures above 50 °C, and the inhibitor protein was totally inactive at 70 °C (10.2% inhibition). From the data presented in Fig. 6 it was inferred that the protease inhibitor was moderately heat stable as there was decrease in activity after preincubation at temperatures above 50 °C. The protease inhibitor was observed to be stable for 2 h at 30 °C (54% inhibition), 40 °C (53% inhibition) and 50 °C (46% inhibition). Further, it was also noted that at temperatures above 50 °C the protease inhibitor was inactive with a decline in inhibitory activity and was stable only for 30 min.
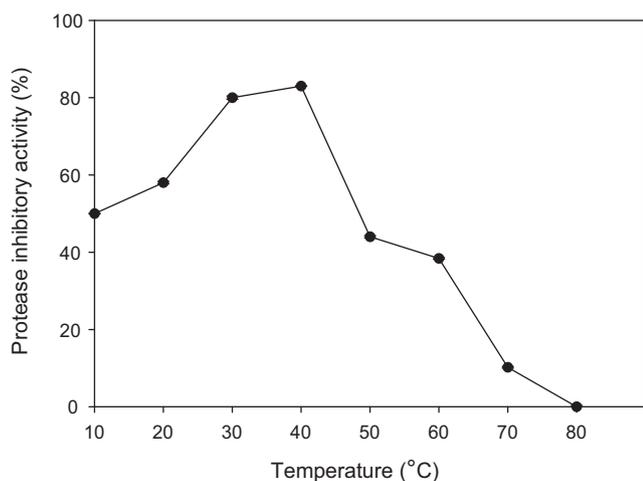
3.3.5. Effect of stabilizers on thermal stability of protease inhibitor

Since the protease inhibitor showed thermal inactivation at 60 °C and 50 °C, effect of additives as thermal stabilizers of protease inhibitor was studied at the same temperatures using glycine, cysteine hydrochloride, PEG 6000, glycerol, sorbitol, casein, $CaCl_2$, urea, sucrose, BSA, and starch. Data presented in Fig. 7 indicated that in general, all the stabilizers promoted thermal stability and inhibitory activities compared to that of control (in the absence of any stabilizers). At 50 °C, maximal stability was promoted by $CaCl_2$ (73% inhibition) followed by BSA (70% inhibition) and sucrose (58% inhibition). At 60 °C, BSA (50% inhibition) followed by $CaCl_2$ (45% inhibition) supported stability compared to that of control (in the absence of any stabilizers). Sucrose and starch, which supported stability at 50 °C, did not promote stability at 60 °C. Whereas, glycerol (31% inhibition) and sorbitol (36% inhibition) effected moderate thermal stability compared to $CaCl_2$ and BSA. PEG 6000, casein, and cysteine hydrochloride were observed to support partial stability at 50 °C and 60 °C while sucrose, urea, starch, and

Fig. 5. Effect of temperature on the activity of protease inhibitor: protease inhibitor assay was carried out at pH 7 with incubation temperature ranging from 10 °C to 80 °C.
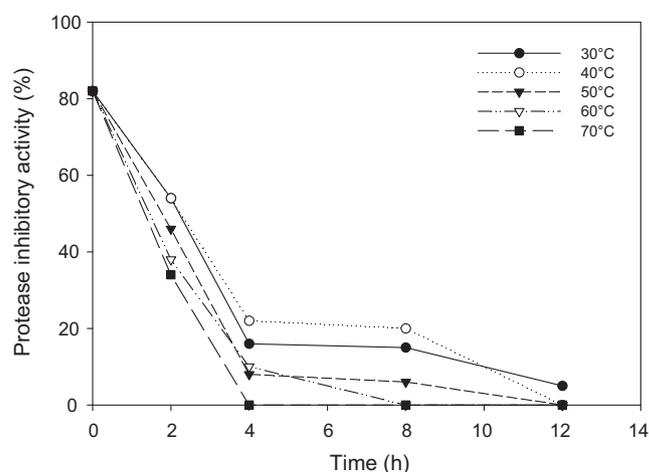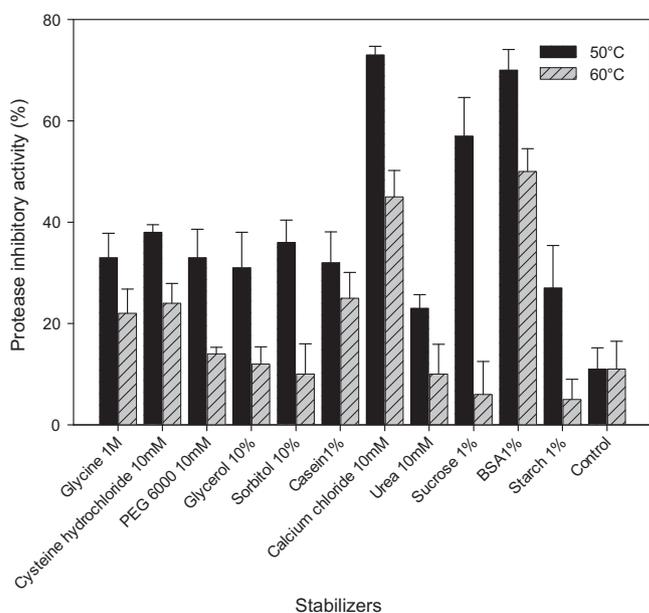

Fig. 7. Effect of stabilizers on the activity of protease inhibitor at 50 °C and 60 °C: protease inhibitor was incubated with different thermostabilizers and inhibitor activity was measured after 4 h of incubation.
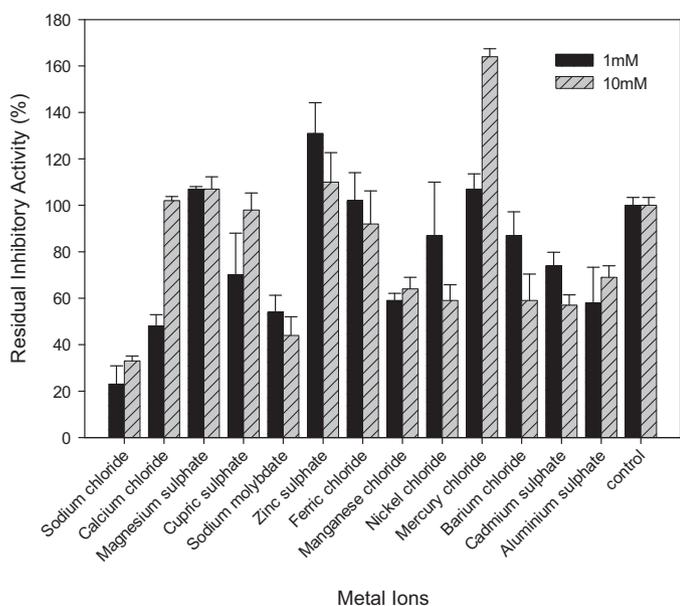
Fig. 8. Effect of metal ions on protease inhibitor activity: protease inhibitor activity was measured after incubating with different concentrations of metal ions for 30 min at 37 °C.

3.3.6. Effect of various metal ions on protease inhibitor activity

The activity profile of protease inhibitor in the presence of different monovalent and divalent metal ions was determined by incubating with different concentrations of metal ions. From the results presented in Fig. 8 it was noted that zinc sulphate which provided divalent Zn^{2+} ions at a concentration of 1 mM enhanced the protease inhibitor activity up to 31% showing a residual inhibitory activity of 131% when compared to control (inhibitory activity considered as 100% in the absence of any metals). Whereas, Hg^{2+} at 10 mM concentration was observed to enhance residual protease inhibitor activity to 164% compared to control. Ca^{2+} and Mg^{2+} at 10 mM concentration also were noted to enhance the protease inhibitor activity only up to a marginal level. Results indicated that presence of Na^{2+}, Ba^{2+}, Ni^{2+} Cd^{2+}, Mo^{6+} and Al^{3+} had a negative effect and did not support protease inhibitor activity when compared to control.

3.3.7. Effect of surfactants, oxidizing agents, and reducing agents on protease inhibitor activity

Result presented in Fig. 9 indicated that all the ionic and non-ionic surfactants tested, except SDS, had a negative effect on the protease inhibitor activity. But interestingly it was found that in the presence of SDS the residual activity of the protease inhibitor was enhanced by 62% compared to control (inhibitory activity considered as 100% in the absence of surfactant). Whereas Triton X 100, Tween 80, and Tween 20 were observed to reduce (50% loss in activity) the activity of protease inhibitor when compared to the activity of control. It was also noted that Brij 35 could inactivate the inhibitor with 67% loss in inhibitory activity.

Data presented in Table 3 indicated that the protease inhibitor activity decreased along with increase in the concentration of oxidizing agents. Thus it was noted that, the inhibitory activity was decreased to 28% and 12% respectively for 1% and 5% concentrations of DMSO. In the same way the inhibitor activity was observed to decrease in the presence of 1% and 5% H_2O_2 to 52% and 32% respectively, compared to control (inhibitory activity considered as 100%
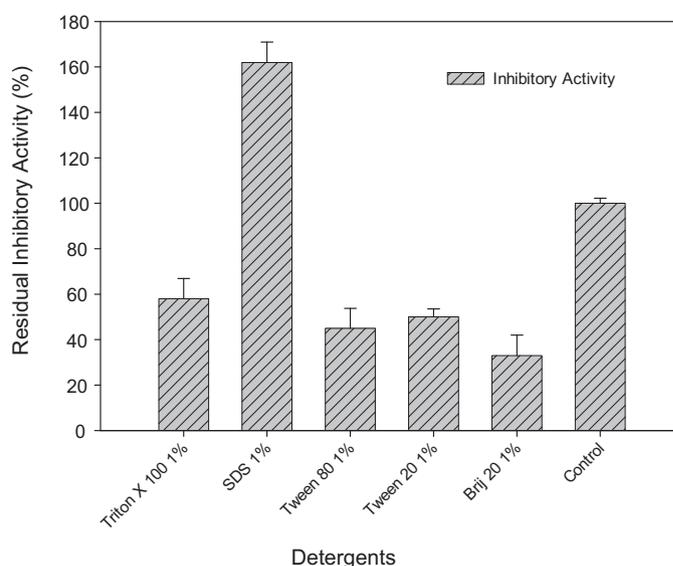

Fig. 9. Effect of surfactants on protease activity: protease inhibitor activity was measured after incubating with different surfactants for 30 min followed by dialysis in phosphate buffer.

in the absence of any oxidizing agent). Reducing agents evaluated for their impact on the activity of protease inhibitor indicated that they could enhance inhibitory activity (Table 3). It was also noted that the inhibitory activities increased along with increase in the concentrations of dithiothreitol (49% enhancement in inhibition at 1%) and β-mercaptoethanol (41% enhancement in inhibition at 1%).

3.3.8. Chemical modifications of amino acids in protease inhibitor

Three different amino acids were individually modified using specific chemical modifiers and the effect of amino acid modifications on protease inhibitor activity was determined. From the results presented in Table 4 it was inferred that PMSF and DEPC led to an enhancement in the inhibitory activities of the protease inhibitor. Further, increase in the concentrations of PMSF (285% at 25 mM) and DEPC (155% at 10 mM) were found to result in an enhancement of protease inhibitor activities compared to control (inhibitory activity of untreated protease inhibitor molecule). In contrast, lysine modification by succinic anhydride was observed to result in the loss of protease inhibitor activity and at higher concentration the protease inhibitor was totally inactive.

Infra red spectrum of native and modified protease inhibitor was analyzed to know whether the enhancement in protease inhibitor activity was due to the structural changes in the protein. From the results obtained (data not shown) it was inferred that a structural change in the carbon–hydrogen bond of the functional group present in the reactive site of the inhibitor has led to a chemical modification of the respective amino acids.

3.3.9. Stoichiometry of protease–protease inhibitor interaction, IC_{50} value of protease inhibition and kinetic studies

The data obtained for the studies conducted on protease–protease inhibitor interaction is depicted in Fig. 10. Extrapolation to zero protease activity (100% inhibition) corresponds to 1.5 nM of inhibitor. It is predicted that the stoichiometry of trypsin–protease inhibitor interaction is 1:1.5 and 35.4 μg of protease inhibitor is necessary to completely inactivate 23.8 μg of trypsin. It was also found that the amount of inhibitor needed for 50% inhibition (IC_{50}) of trypsin calculated from the graph was 0.6 nM.

Data obtained for the kinetic studies performed with trypsin indicated that the protein protease inhibitor molecule has a

Table 3
Effect of oxidizing and reducing agents on protease inhibitor activity.

	Chemical compound	Concentration (%)	Residual inhibitory activity (%)
Oxidizing agents	Dimethyl sulfoxide (DMSO)	0	100
		1	28
		2	28
		3	19
		4	18
		5	12
	Hydrogen peroxide (H_2O_2)	0	100
		1	52
		2	46
		3	44
		4	38
		5	32
Reducing agents	Dithiothreitol (DTT)	0	100
		0.2	100
		0.4	102
		0.6	129
		0.8	136
		1.0	149
	β-Mercaptoethanol	0	100
		0.2	108
		0.4	117
		0.6	120
		0.8	124
		1.0	141

Table 4
Effect of chemical modifications of specific amino acids in the protease inhibitor molecule on protease inhibition activity (activity expressed as percent residual protease inhibitor activity).

S. No	Concentration tested (mM)	Succinic anhydride on lysine[a]	Diethyl pyro-carbonate (DEPC) on histidine[b]	PMSF on serine[c]
1	0	100	100	100
2	5	70	100	195
3	10	80	155	240
4	15	60	160	255
5	20	85	160	280
6	25	90	165	285

[a] Reaction conditions: 30 °C, 0.1 M sodium carbonate buffer (pH 8.0), 120 min.
[b] Reaction conditions: 30 °C, 0.1 M Tris/HCl buffer (pH 7.0), 30 min.
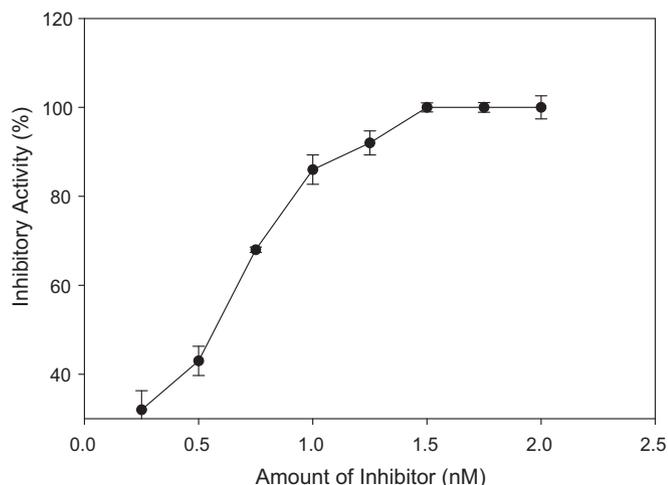[c] Reaction conditions: 25 °C, 0.5 M Tris/HCl buffer (pH 7.8), 120 min.

reversible mechanism of action. It was observed that identical concentration of trypsin (1.0 nM) preincubated with enzyme buffer alone and with different concentrations of inhibitor (4, 6 and 8 nM) yielded different slopes for plots $1/v$ versus $1/[s]$ for nine different $[s]$ values (Fig. 11). Inhibition of substrate hydrolysis occurred at very low concentration of protease inhibitor and the K_i was calculated from the Line Weaver–Burk plot, which was found to be

1.5 nM under the assay conditions. The low K_i value implies that it is a possible powerful inhibitor of serine proteases.

4. Discussion

Most of the natural protease inhibitors are proteinaceous in nature and are located mainly in seeds, leaves, and tubers which act as specific defense and regulatory proteins. In plants the presence of protease inhibitors are mainly considered as storage proteins and as defense tools [34]. Many reports are available on the isolation, purification and characterization of protease inhibitor from seeds of legume plants [5]. Although *M. oleifera* is known as source of several low molecular weight bioactive constituents with pharmaceutical and industrial applications [35], it is not reported so far as the source of any protein inhibitors. Results obtained in the present investigation indicated that *M. oleifera* is a potential source for protease inhibitor since it recorded 76% protease inhibitor activity. Evaluation of the distribution of protease inhibitor in different plant tissues of the mature *M. oleifera* plant revealed that the mature leaves contained protein molecules that could effect maximum percent of protease inhibition followed by the seeds. Further, the distribution of inhibitor in flowers, roots, and bark were negligible when compared to the leaves and seeds. Since leaves are the major tissues attacked by pest and pathogens, the accumulation of this protease inhibitor was maximal in leaves compared to other parts indicating a tissue specific expression of these proteins. Hence, it may be presumed that leaves and seeds of *M. oleifera* are rich source

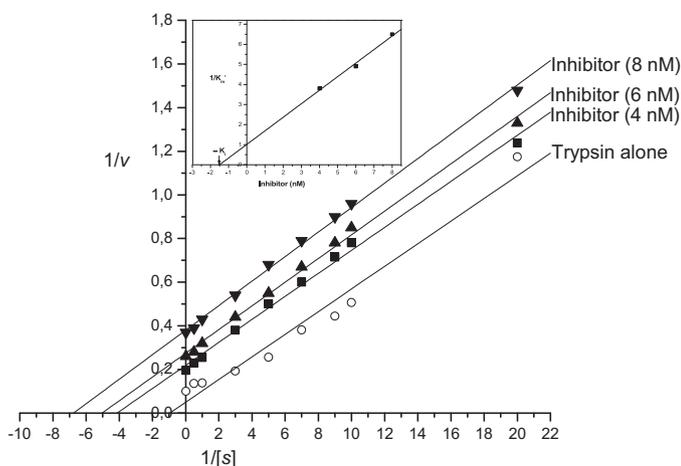**Fig. 10.** Stoichiometry of protease–protease inhibitor interaction.

Fig. 11. Lineweaver–Burk plot analysis of the inhibition of trypsin by *M. oleifera* protease inhibitor: the incubated enzyme reaction mixture contained various concentrations of inhibitor as indicated. The rates of each reaction were calculated and the reciprocal values of these velocities are plotted against reciprocal concentrations of trypsin as a Lineweaver–Burk plot. The values of $1/K_m$ derived from the double reciprocal plot are plotted against the relevant concentrations of protease inhibitor in order to derive the K_i value for *M. oleifera* protease inhibitor (*inset*).

of protease inhibitor, which is mostly directed towards serine proteases such as trypsin and chymotrypsin. The extraction medium has a major role in the complete extraction of the protein from any desired source. Hence, different solvents were used for extracting proteinaceous protease inhibitors from the leaves. Protein concentration and protease inhibitor activities were maximal in the extract prepared with phosphate buffer, which facilitated complete release of the proteins from the leaves into the solvent. In fact 0.1 M phosphate buffer (pH 7.6) was reported to be a good extractant for the maximal extraction of proteins from *Cajanus cajan* seeds with high amount of trypsin inhibitory activity and protein concentration [20].

The results of SDS-PAGE analysis of the purified protein protease inhibitor molecule undoubtedly evidenced the homogeneity of protease inhibitor. An apparent molecular weight of 23.6 kDa was obtained in SDS-PAGE. Reverse zymography on gelatin-incorporated polyacrylamide gel and dot-blot assay confirmed inhibition of gelatin hydrolysis to a greater extent. It was reported earlier that the molecular mass of Kunitz type inhibitors are in the range of 18–26 kDa and are mostly monomeric or dimeric members, in which subunits are linked by a disulphide bridge [36]. Hence it is concluded that the protease inhibitor isolated from *M. oleifera* is a small protein with a molecular mass of 23.6 kDa and belongs to the Kunitz type of serine protease inhibitor family. The amino acid composition of the purified inhibitor sample indicated the presence of high amount of glycine (27.29%) and low amount of lysine (0.22%). Since the glycine residues lack a side-chain, they can avoid steric clashes encountered by other amino acids which leads to an increase in the number of accessible conformations in the unfolded states [37] giving stability to the protease inhibitor.

According to Mello et al. [38], most inhibitors in the Kunitz family are acidic and some are very sensitive to acidic pH and stable in the alkaline pH. It is reported that the intra molecular disulphide bridges are presumably responsible for the functional stability of Kunitz type protease inhibitors in the presence of physical and chemical denaturants such as temperature, pH and reducing agents [39]. The observations made with the protease inhibitor from *M. oleifera* that it was totally inactive in the extreme acidic and basic pH conditions and was active and stable in the pH range 5–10 are in agreement with their report. Probably the extreme pH conditions could have totally altered the structure of the inhibitor

such that they no longer bind with the enzymes or with their substrates. Under strong acidic or alkaline conditions, the proteinaceous inhibitors get denatured and as a consequence they lose their activity partially or completely. In general, all the protease inhibitors isolated from plants have a wide pH range of 2–10. Many enzyme inhibitors in seeds are present in multiple molecular forms, which may differ considerably in their pI values. The majority of the protease inhibitors exhibiting anti-feedent properties reported so far is active against the neutral serine proteases such as trypsin and chymotrypsin [5]. Hence, the stability of protease inhibitor of *M. oleifera* in a wide range of pH signifies its possible use as biopesticide, which can withstand highly alkaline conditions of insect's gut flora. Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material.

Most of the inhibitors of Kunitz family plant protease inhibitors are active at temperatures up to 50 °C [40]. In the present study the highest optimal temperature recorded for maximal activity of *M. oleifera* protease inhibitor was in the range of 30–40 °C. However, the protease inhibitor activity declined at temperatures above 50 °C and was totally inactive at 80 °C. Thermal inactivation of protease inhibitor at different temperatures resulted in a progressive loss of activity at temperatures above 40 °C. The inhibitor is moderately stable up to 2 h at 30 °C and 40 °C with 50% protease inhibitor activity. These observations indicate that the protease inhibitor has high intrinsic stability in its native state, which gives a high degree of thermal stability. This property of protease inhibitor is typical of all trypsin inhibitor family. Soybean trypsin inhibitor purified by gel permeation chromatography had much thermal stability and the presence of a protein substance accelerated the thermal inactivation of the inhibitor [41]. The high thermal and pH stability of *M. oleifera* protease inhibitor testified its applications in various industries.

Enhancement of thermal stability is desirable for most of the biotechnological applications of proteins. Naturally occurring osmolytes such as amino acids, polyols and salts are known to protect proteins against thermal inactivation by stabilizing the thermally unfolded proteins [42]. Thermal stability increases the efficiency of proteins and is one of the essential features for their commercial exploitation [43]. Among the stabilizers tested almost all of the stabilizers attributed to the stability at 50 °C and promoted the inhibitory activity compared to the control. Maximal stability was provided by $CaCl_2$ (73%) followed by BSA (70%). At 60 °C, BSA promoted maximal protease inhibitor activity than $CaCl_2$, whereas the stabilizers such as sucrose and starch, which supported the stability at 50 °C, did not promote the inhibitory activity.

Protein stability is related to the increase of hydrophobic forces inside these molecules [44]. Solvents with a low dielectric constant strengthen the hydrophobic interactions among non-polar residues [45]. In the presence of such solvents several proteins show a greater resistance to thermal denaturation processes [46,47]. In the present study $CaCl_2$ provided a significant increase in the stability of protease inhibitors at higher temperatures. Ca^{2+} ions stabilize the protein through specific and non-specific binding sites and may also allow for additional binding within the protein molecule preventing the unfolding at higher temperatures. High content of charged residues present in the protease inhibitors may contribute considerably towards maintaining stability by electrostatic interaction in neutral pH [48].

Protease inhibitor (API-1) isolated from actinomycete was reported to improve the thermal stability of protease of fungus *Conidiobolus macrosporous* that has potential biotechnological applications in both detergent and leather industries [43]. Thus thermal inactivation of detergent proteases can be prevented by binding of protease inhibitor. Surfactants are used extensively for solubilizing protein from lipid membranes and other biological

materials, and for maintaining the solubility of certain proteins in the solution. Protease inhibitors and surfactants are routinely used together in cell lysis buffers to inhibit unwanted proteolysis and facilitate membrane protein solubilization in protein purification procedures. Studies on the effect of surfactants on the activity of protease inhibitor from *M. oleifera* indicated that SDS enhanced 62% protease inhibitor activity compared to control. Hence, it is suggested that SDS can act as stabilizer for the protease inhibitor under unfavorable conditions like thermal denaturation. The reduction in the protease inhibitor activity observed in the presence of other surfactants may be attributed to the combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and the direct interaction with the protein molecule. Whereas, the hydrophobic nature of SDS apparently caused rearrangement of peptide backbone conformation leading to helix-formation with more hydrophobic residues exposed and consequently available to associate with the surfactants [49].

Metal ions have a major role in maintaining the structural integrity of protease inhibitor. The side chain carboxylates of glutamate and aspartate residues can participate in binding of divalent cations to metalloproteins. Studies on the impact of metal ions on the activity of *M. oleifera* protease inhibitor illustrated that addition of divalent ions such as Zn^{2+} at a concentration of 1 mM enhanced the protease inhibitor activity up to 31% and Hg^{2+} at a concentration of 10 mM enhanced up to 64% of protease inhibitor activity compared to that of control. Ca^{2+} and Mg^{2+} at higher concentration (10 mM) enhanced the protease inhibitor activity only to a marginal level. This observation implies the role of metal ions in the activity of *M. oleifera* protease inhibitor. The metal ions will bind to the protein in a reversible mechanism and they retain their conformational stability for their biological activity.

From the results obtained it was inferred that the activity of *M. oleifera* protease inhibitor was affected by the increase in the concentration (from 1% to 5%) of oxidizing agents DMSO and H_2O_2 and consequently the activity declined rapidly. The loss in activity may be attributed to the probable oxidation of methionine residue present at the reactive site of the inhibitor [50]. Thus oxidation could be a major factor for the regulation of protease inhibitor activity. With respect to the reducing agents it was observed that the activity of *M. oleifera* protease inhibitor increased along with increase in concentrations of dithiothreitol. The intra molecular disulphide bridges are presumably responsible for the functional stability of Kunitz type protease inhibitor in the presence of reducing agents. Dithiothreitol at a concentration of 1 mM had no effect on the activity or stability of *Peltophorum dubium* protease inhibitor [36]. *Erythrina caffra* trypsin inhibitor, a Kunitz type trypsin inhibitor from *E. caffra* retained its inhibitory activity after reduction with dithiothreitol [51].

In proteins, the arrangement of functional groups in a particular manner is an essential requirement for their activity. Knowledge on the reactive site and the functional group involved for the particular biological activity would facilitate easy designing of novel protease inhibitors as drugs for blocking specific protease actions. The modification of amino acids by chemicals is a valuable tool for identifying the reactive site and understanding of the chemical group involved in specific protein–protein interactions. In the present study the results obtained for the effect of chemical modifiers on the activity of *M. oleifera* protease inhibitor indicated that modification of amino acid serine resulted in enhanced inhibitory activity to a great extent. The IR spectrum obtained for native and modified protease inhibitor suggested possible modification in the amino acid present at the reactive site. The loss of activity after modification may be due to the change in the conformation of the inhibitor molecule during binding of the modifying agents. Alternatively, one of the modified residues, though not involved in the reactive site of the inhibitor,

may disturb the interaction of the inhibitor with the target enzyme.

In chemical modification, a chemical reagent binds covalently to specific amino acid side chains of a protein and may produce changes in the properties/activity of a protein. Attempts to correlate these changes with catalytic activity have been made previously [52,53]. In the majority of specific serine protease inhibitors, the inhibitory action is localized to a specific reactive site situated within a loop closed by a disulphide bridge [54]. It is reported that chemical modification of Pearl millet cysteine protease inhibitor provided evidence for the presence of two distinct sites responsible for antifungal and antifeedant activities [55]. *P. dubium* protease inhibitor, a Kunitz type serine protease inhibitor, was inactivated by lysine and arginine modification with tri-nitrobenzene-sulfonic acid and 1,2-cyclohexanedione respectively [36].

Protease inhibitors from plants and microorganisms are characterized by either a reversible or irreversible mechanism [56]. Kinetic studies of trypsin by *M. oleifera* protease inhibitor revealed that it has a reversible mechanism of action. It was observed that the stoichiometry of trypsin and protease inhibitor interaction takes place in a 1:1.5 molar ratio and the amount of protease inhibitor needed for the 50% trypsin inhibition was 0.6 nM. It may be noted that in a non-competitive inhibition, the inhibitor binds to the enzyme and does not compete with the substrate (competitive inhibitor) for the active site. Instead, it will bind to an allosteric site where it will produce changes in the enzyme's structure and the substrate cannot bind to the changed active site. In this case, K_m remains the same whereas V_{max} changes. Results of the kinetic studies of protease inhibition obtained in the present study and presented in the Lineweaver–Burk plot indicated that trypsin inactivation occurs by uncompetitive inhibition during which the inhibitor binds only to the enzyme–substrate complex and as a result both affinity of the enzyme (K_m) and V_{max} undergoes change. Further the inhibition of substrate hydrolysis occurred at very low concentrations of protease inhibitor and the K_i was calculated to be 1.5×10^{-9} M under the assay conditions. The low K_i value indicated a relatively high affinity of *M. oleifera* protease inhibitor for the trypsin. The inhibitory activity of Kunitz type protease inhibitor varies with the binding enzyme. A few members of this family are specific for chymotrypsin but do not inhibit trypsin. Some are specific for both trypsin and chymotrypsin with high affinity. *P. dubium* protease inhibitor inhibited bovine and porcine trypsin stoichiometrically (K_i of 4×10^{-10} M and 1.6×10^{-10} M respectively) but affected bovine chymotrypsin only weakly (K_i of 2.6×10^{-7} M) [36].

5. Conclusions

M. oleifera have been recognized as a source of several bioactive substances and in waste water treatment, but so far it was not reported as a source of protease inhibitor. In our study the potential of this plant as a source of serine protease inhibitor is indicated. The characterization studies conducted on various aspects strongly advocates the probable use of this inhibitor against therapeutic proteases, as biocontrol insecticides, and probable protectant against proteolytic bacteria in food preservation. We conclude that *M. oleifera* protease inhibitor is a valuable source of a potential serine protease inhibitor with immense application in biotechnology and in biomedical field.

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